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Effect of P16ink4a Immunohistochemistry on Screening of Carcinoma Cervix

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ABSTRACT

Background: Currently the primary screening test for cervical cancer in most countries is Papanicoaou cytology. But sensitivity with cervical cytology is low (approximately 72% %) . Detecting the viral DNA is a highly sensitive test for early detection of cervical cancers and precancerous lesions. But it is costly and can detect presence of virus but not the cellular alterations. p16INK4a is a specific biomarker helping in detection of transforming HPV disease . Also it can differentiate non-neoplastic from low grade lesions and also low and/or high grade lesions from neoplastic ones. The objective of this study is to detect overall positivity of p16INK4a immunohistochemistry in suspected cases of cancer cervix and compare it with other methods of screening like cytology and histopathology.

Material and Methods: One hundred patients with strong clinical suspicion of carcinoma cervix were included in the study. In all patients, papanicolaou smear test, biopsy and p16INK4a immunostain were done. The results of cytology was compared with histopathology and p16 immunostain and analyzed to find out the accuracy of each method alone and in combination by taking histopathology as gold standard.

Results: 70% cases were abnormal in cytology, out of which 66 were abnormal in biopsy comprising of 38% precancerous lesions and 28% carcinoma. All cases with carcinoma in papanicolaou smears, were proved as carcinoma on biopsy and also were positive for p16INK4a. But in precancerous lesions like CIN 1/2, there was discrepancy between biopsy and IHC. p16INK4a was positive in 5.6% (4/70) patients with abnormal pap and normal biopsy. But by combining IHC with biopsy the specificity and the positive predictive value of pap test was increased from around 80 % to 100%.

Conclusion: There is a definite place of p16INK4a IHC in diagnosing and deciding the treatment modalities of low grade cervical precancerous lesions with ambiguous biopsy results.

KEYWORDS cervical cancer screening, pap smear, biopsy, p16INK4a.

Introduction:

Cervcal cancer is the third most common cancer and the fourth most common cause of cancer related deaths in female population.[1] In India, it is the leading cause of cancer related deaths among females between 15 and 44 years of age. The cause is lack of proper screening methods and absence of periodical and routine screening. Papanicolaou smears is a cost effective and rapid screening technique for diagnosing precursor lesions of cervical carcinoma. But it is associated with high false positive (30%) and false negative (15-50%) results due to subjective variation in observation [2,3]. Also, sensitivity with single test of cervical cytology is between 55 to 80% with many equivocal results for which it needs multiple visits for diagnosis and treatment and need for further work up [4,5] .Therefore pap cytology-based screening is frequently found unsuitable and challenging in low-resource settings [6]. Histopathology is considered gold standard method in diagnosis of precancerous and cancerous lesions of cervix. But this also has the bias of interobserver variability[7].

Recently tests for presence of viral DNA has emerged as a highly sensitive test for early detection of cervical cancer, more frequently detecting premalignant lesions than pap cytology. In many industrialized countries , it is used in addition to pap cytology and currently in certain settings as an alternative for primary screening [8]. However, detecting HPV DNA is a poorly specific test for real cellular alterations in context of the high prevalence of HPV infections, particularly in young women. So this also requires additional triage tests for the specific segregation of women needing further work-up or treatment.[9] And in poor and developing countries, performing tests for detection of HPV DNA is not feasible.

Because of this difficulties in screening of cancerous and precancerous cervical lesions, different biomarkers have been identified . These can reduce false negative results, multiple visits, unnecessary treatments and finally costs. One promising marker is the cyclin dependent kinase inhibitor p16INK4a(p16), which becomes overexpressed in response to

viral oncogene E7 expression. [10]

A number of studies have investigated the clinical use of p16INK4a as a specific biomarker for cells with transforming HPV infections. [11] HPV-transformed cells over express p16INK4a but retain the capacity to proliferate. Because this protein is not expressed in the normal cervical epithelium, p16 over expression allows to specifically identify dysplastic lesions and will reduce interobserver disagreement of conventional histological or cytological tests.[12]

In the present study we want to know the effect of immunohistochemistry (p16INK4a) on the cervical cancer screening methods and if it can help in earlier diagnosis and treatment of patients with ambiguous histopathological reports.

Material and Methods:
The present study was conducted over a period of two years in Department of Obstetric and Gynecology, and Department of Pathology. One hundred patients were included in the study. There was a strong clinical suspicion of carcinoma cervix or its precursors from history and cervical findings in all the women. Informed consent was taken from all the patients. This study has been approved by institutional ethical committee.

History taking, clinical examination, per speculum examination and pap smear collection were done in all the patients. The pap cytology technique followed the conventional procedures of smear taking with Ayre's spatula, fixation with 95% ethanol and staining with papanicolaou stain. Cervical cytology reporting was done as per the current system of Bethesda reporting 2001.[13] Cervical biopsies were taken in all the cases. All samples were fixed in formalin and embedded in paraffin wax by conventional histotechniques. H&E stained slides of all samples were reviewed by two histopathologists. In cases of controversy, both used to sit together and examine in pentahead microscope and a common consensus was taken. In both cytology and histopathology, low grade squamous intraepithelial lesion (LSIL) and cervical intraepithelial neoplasia 1(CIN1) will be used interchangeabily. Similarly, high-grade squamous intraepithelial lesion (HSIL) and cervical intraepithelial neoplasia 2/3 (CIN2/3) will also be used to denote same condition. p16INK4a immunohistochemical staining was done in all the paraffin blocks using the similar protocol used by Murphy N.[14] p16 immunostained slides were reviewed, and strong nuclear as well as cytoplasmic staining was considered a positive reaction. The distribution of p16 INK4a positivity was scored on a semi quantitative scale, as follows: negative (< 1% of the cells were positive), sporadic (isolated cells were positive, but < 5%), focal (small cell clusters, but < 25% of the cells were positive) and diffuse (> 25% of the cells were stained).[15]

Stastistical analysis was performed using SPSS 17 software, significance between two proportion were analysed using Z test, ODDS ratio was calculated using bivariate analysis.

Result:
One hundred patients attending Gynaecology outpatient department with strong clinical suspicion of carcinoma cervix or its precursors from history and cervical findings were included in this study. The mean age of presentation was 46.14 ±11.85 years with age range of 30-70 years. The mean age at marriage, parity and duration since last child birth were 16.98 ±2.11 years, 3.88±1.67 and 14.28±10.34 years respectively. Thirty four percent patients attained menopause with the mean duration of menstrual cycles of 15.76 ± 9.4 years at presentation (Table-1). Abnormal bleeding was the most common mode of presentation in both premenopausal and postmenopausal patients [81.81% vs 88.23%,p=0.40], followed by abnormal vaginal discharge [66.66% vs 58.82% ,p=0.43]. Factors like age>40 years, early age at marriage (<16yr) and multiparity (>4) were strongly associated with carcinoma cervix (Table-2).

On taking biopsy as the reference gold standard, the sensitivity of cytology was 84.62% , specificity was 81.82 % , positive predictive value (PPV) was 94.28 % and negative predictive value (NPV) was 60 % respectively. On taking immunohistochemistry (IHC) as the gold standard, sensitivity, specificity, PPV and NPV of cytology were 93.3% , 65 %, 80 % and 86.67 % respectively. When both biopsy and IHC were taken as reference standard, specificity and PPV of cytology became 100 %, sensitivity was 85 %, and NPV was 60%.[Table-4]

Cytology was negative for intraepithelial lesion/malignancy in 40 cases (normal in 30 cases and reactive changes suggestive of inflammation were seen in 10 which will be denoted as inflammatory smears). Out of 30 cases, biopsy and immunohistochemistry were normal in 18 (60%) cases and the remaining 12(40%) cases were found to be LSIL in eight samples in biopsy which were negative in IHC and four cases were adenocarcinoma showing diffuse staining pattern on IHC . LSIL was found in 18% of cases having 10 LSIL on biopsy; in IHC four came to be negative , four sporadic and two focally positive. HSIL (CIN 2) was seen in four biopsy samples and all were positive in IHC i.e. two were sporadic and two focally stained. HSIL(CIN 3) was also detected in four samples where two were sporadic and two focal on IHC(Fig1a&b). Invasive squamous cell carcinoma was seen in 28 cases in biopsy and out of which 24 were diffusely positive but four stained focally in IHC(Fig2a&b).[Table-5]

Discussion: p16 INK4a is a tumor suppressor protein (cyclin dependant kinase inhibitor). It acts as a negative regulator of cell cycle progression and differentiation by controlling the activity of tumor suppressor protein pRb. In a cell with transforming HPV infection the viral oncogenes ,especially E7 disrupts the protein of retinoblastoma (pRb) from its binding to E2F transcription factor. By doing this, it promotes cell cycle progression. This HPV E7 combination causes continuous inactivation of Rb due to absence of a retinoblastoma protein (Rb)-dependent negative feedback loop and results in increased p16 levels. Over time, p16 accumulates in the nucleus and cytoplasm of affected cells and can be detected by immunostaining.[16] Therefore, increased p16 levels may reflect HPV-induced dysplasia with deregulated E7 expression.

Histopathological confirmation of SIL plays a critical role in clinical management of preinvasive cervical diseases. There are well-defined criteria for histopathological diagnosis of CIN. But, sometimes it is difficult to distinguish both low- and high grade lesions from their mimics.[17,18,19] The distinction of HPV induced alterations from florid reactive changes, immature metaplastic lesions, and atrophic changes may pose problems. In these instances biomarkers can help in distinguishing CIN from other non-neoplastic cervical lesions, to prevent under treatment or overtreatment .[20,21] Also positive staining for a biomarker in cervical cells can establish it as a dynamic (transforming HPV infection) process where aggressive treatment is needed. But the conventional haematoxylin and eosin (H&E), gives a false impression of a static process since it can not identify whether HPV infection is in proliferative or transforming status. Correlation has been reported between HR-HPV oncogene expression and high scores of p16 positivity . [22] Addition of a consecutive p16-stained slide to the HE stained slides has been shown to improve significantly interobserver agreement in cervical biopsies and to help in the identification of asymptomatic precancerous lesions. [23,24,25,26] Also with the use of p16, lesion grading can be faster, especially concerning aggressive-appearing low-grade lesions, which otherwise might be upgraded. [24]

Therefore this study was undertaken to identify the presence of p16 in precancerous and malignant leisons of cervix in addition to quntify the intensity and area of staining in these cells.

In the present study factors like age>40 years, early age at marriage (<16yr) and multiparity (>4) were strongly associated with carcinoma cervix similar to previous studies. [14,15] In

premenopausal age group, intermenstrual bleeding was more common than postcoital bleeding.

There were 28 cases of CIN 1, 10 cases of CIN2 and 8 cases of CIN3 in histopathology. Out of them in majority (12/28) cases IHC was negative but was found to be positive in all cases of CIN2 and CIN3 (18/18). This was also observed by Klaes R et al whi noted absent or sporadic immunostain in 9 out of 47 cases (19%) of CIN1 lesions. [15] p16INK4a was positive (diffuse and strong) in all cases of invasive carcinoma. This data is comparable with the above authors who found positive immunostain in 100% cases of HSIL (CIN2 and CIN3) and 58/60 cases of invasive carcinoma. All negative immunostain was found in precancerous lesions mostly CIN 1.

There were four cases of adenocarcinoma, cytology was normal but biopsy came out to be adenocarcinom and p16 was diffusely positive. The cause of discripancy between cytology and biopsy may be failure to detect adenocarcinoma cells either due to abundant mucin in background or the well differentiated glandular cells might have been missed during evaluation. This study determines the significance of IHC in detection of glandular malignancies. Negri et al. in 2003 conducted a study to determine whether immunostaining of p16 is useful in detecting adenocarcinomas of cervix and its precursors in histologic and cytologic specimens.[27] A total of 45 patients with glandular lesions including 18 cases of adenocarcinoma in situ(AIS), adenocarcinoma (n=8), endocervical glandular atypia (n=4) and reactive (n=15) lesions were identified. P16 was detected immunohistochemically in all 26 cases of AIS and adenocarcinoma (100%). Compared with HPV DNA detected by in situ hybridization, p16 immunohistochemistry appears to be more sensitive and easier to perform, method for distinguishing endocervical from endometrial adenocarcinomas. In the present study also all cases of adenocarcinoma showed p16 positivity. This finding also suggested that these adenocarcinomas were endocervical but not endometrial as p16 positivity in endometrial adenocarcinoma is less diffuse and less intennse.

On taking biopsy as the reference gold standard, the specificity of cytology was 81.82 %, and the positive predictive value was 94.28% while combining IHC with biopsy the specificity and the positive predictive value of pap test was increased to 100% which was also observed in study done by Qi Zhang et al. [28] Thus, there is some evidence that diffuse p16 immunostaining in histological specimens could be a predictor of disease progression identifying those low grade lesions (CIN1) that need more intensive follow-up. [29,30,31]

In the present study about one third of normal cytology cases had abnormal biopsy and all of them were negative on IHC, implying that biopsy overestimated cytology in these cases, similar to studies done earlier by Hariri J et al where the negative predictive value of p16 to predict the outcome of the cases of CIN 1 is as high as 96%, strongly suggesting an important role of p16 in the assessment of this type of lesions. [29] All high grade lesions showed p16 (INK4A) positivity in our study which is at par with study done earlier by Liao GD. [29]

In 5% cases of abnormal cytology, biopsy was found to be normal, but IHC showed focal (CIN-II, CIN-III) and sporadic (CIN-I) staining pattern suggestive of precancerous changes in them. This suggested biopsy underestimated cytology in these cases which was similar to the observation laid by Omori M et al.[31] These patients need regular follow up as disease progression is common in around 10% in CIN-I, CIN-II, and 20% in CIN-III.

Limitations: This study has certain limitations. It is not a population based study so result of this study cannot be extrapolated for entire population. Second the sample size is small, more number of cases are required to draw a definite conclusion.

Conclusion:

p16 INK4A immunohistochemistry revealed that there was a significant over expression and upregulation in different groups and as we move from normal cervical epithelia to dysplasia of varying severity to carcinoma, the p16 positivity was increased.

About one third of cases, biopsy overestimated cytology and in 5% of cases underestimated cytology. The specificity and positive predictive value reached to 100% on adding immunohistochemistry. Thus there is a definite place of p16INK4a IHC in deciding the treatment modalities of low grade cervical precancerous lesions with ambiguous biopsy results.

Table-1. Demographics of the study population.

Age Group	Percentage %	Parameters	Mean±SD in years
30-40 years	48	Age	46.14±11.85
40-50 years	16	Age of marriage	16.98±2.11
50-60 years	26	Parity	3.88±1.67
>60 years	10	Last child birth	14.28±10.34
		Duration of menopause (n-34)	15.76±9.4

Table-2 Association of risk factors with carcinoma cervix

Variables	Percentage	OR	95% CI	pvalue
Age <40 yrs	40%	0.12	0.04-0.40	<0.0004*
40-59 yrs	36%	3.57	1.47-8.62	0.004*
≥60 yrs	24%	1.7	0.6-4.53	0.24
Age at marriage <16yrs	44%	3.05	1.27-7.30	0.012*
Multipara>4	48%	2.38	1.0-5.64	0.048*
OCP use	20%	0.46	0.14-1.5	0.205

*significant values, OCP-Oral contraceptive pills

Table 3.Clinical and histological profile of patients (n=100)

Per speculum examination	Percentage	Cytology	%
Normal	44%	Normal	30
Unhealthy	28%	Inflammatory	10
Erosion	4%	LSIL	18
Growth	24%	HSIL	18
		Squamous cell carcinoma	24
Histology	% (n=100)	Immunohistochemistry P16 INK4a	percentage
Normal	22	Negative	40
CIN-I	28	Sporadic	12
CIN-II	10	Focal	14
CIN-III	8	Diffuse	34
Squamous cell carcinoma	28		
Adeno carcinoma	4		

CIN-Cervical intraepithelial lesion, SIL-Squamous intraepithelial lesion

Table -4: Sensitivity and specificity of different tests for diagnosis of carcinoma cervix

Screening methods	Sensitivity%	Specificity%	PPV%	NPV%
Pap/Biopsy	84.62	81.82	94.28	60
Pap/IHC	93.3	65	80	86.67
Pap/ Biopsy+IHC	85	100	100	60

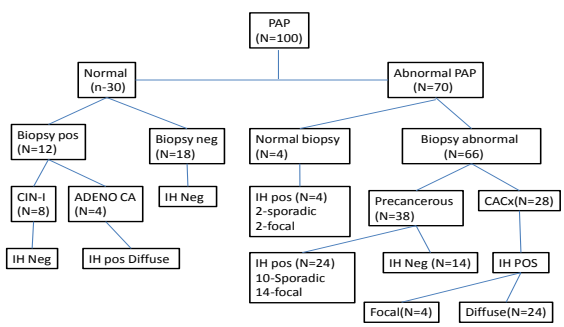
IHC-Immunohistochemistry

Table-5: Correlation between Cytology , Biopsy and Immunohistochemistry

Cytology%	Histology	Immunohistochemistry
Normal (30)	Normal (18)	Normal (18)
	CIN-I(8)	Neg (8)
	Adenocarcinoma(4)	Diffuse (4)
Inflammatory (10)	CIN-I(8)	Neg (8)
	NORMAL(2)	Sporadic(2)
LSIL (18)	CIN-I(10)	Neg (4)
		Sporadic(4)
		Focal(2)
	CIN-II(4)	Sporadic(2)
		Focal(2)
	CIN-III(4)	Sporadic(2)
		Focal(2)
HSIL (18)	NORMAL (2)	Focal(2)
	CIN-I(2)	Focal(2)
	CIN-II(6)	Neg(2)
		Sporadic(2)
		Diffuse(2)
	CIN-III(4)	Focal (2)
		Diffuse(2)
	LCK(2) Focal(2)	
	LCNK(2)	Focal (2)
Invasive carcinoma (24)	LCNK(12)	Diffuse (24)
	LCK(12)	

CIN-Cervical intraepithelial neoplasia, HSIL-High grade intraepithelial lesion, LCK-Large cell keratinising, LCNK-Large cell non-keratinising

Figure-1: Result sheet



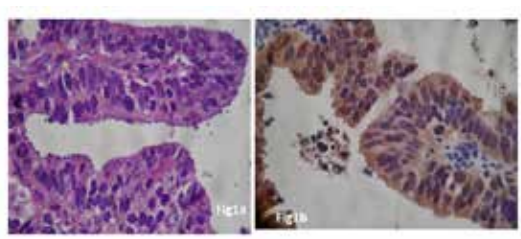


Fig1a&b-H&E & p16 stain in HSIL,X400

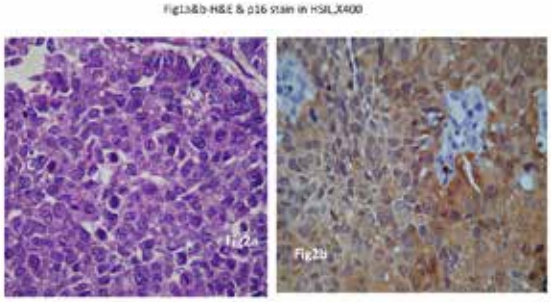


Fig2a&b-H&E & p16 stain in Squamous cell carcinoma,X400

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